Plant Physiol. (1989)89, 452–456
0032-0889/89/89/0452/05/$01.00/00

Received for publication June 7, 1988
and in revised form September 16, 1988

Stabilization of Isolated Photosystem II Reaction Center Complex in the Dark and in the Light Using Polyethylene Glycol and an Oxygen-Scrubbing System[1]

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ABSTRACT

The photosystem II reaction center as isolated (O Nanba, K Satoh [1987] Proc Natl Acad Sci USA 84: 109–112) is quite dilute and very unstable. Precipitating the complex with polyethylene glycol and resuspending it in buffer without detergent concentrates the reaction center and greatly improves its stability at 4°C in the dark as judged by light-induced electron transport activity. Furthermore, a procedure was developed to minimize photodestruction of polyethylene-glycol-concentrated material at room temperature in the light. The ability to stabilize the photosystem II reaction center should facilitate future photophysical, biochemical, and structural studies of the complex.

The similarity between the primary structures of the L-M dimer of the bacterial reaction center and the D1-D2 proteins of PSII (19, 25) suggested an analogous structural and functional relationship between the reaction center of both types of organisms (7, 22). This suggestion was recently supported by the isolation of the D1-D2-Cyt *b*-559 complex from spinach (17) and, more recently, pea (1). This complex contains four to five chlorophylls, two Pheos,[5] one β-carotene, and one to two heme prosthetic groups per reaction center, but it lacks quinones and Mn (17). Nevertheless, photophysical studies demonstrated that the complex was the simplest PSII preparation yet isolated that still performs the following primary photochemical step (6):

$$P_{680} + \text{Pheo} \xrightarrow{h\nu} P_{680}^{+} + \text{Pheo}^{-} \quad (1)$$

where P_{680} is the primary donor. The complex also catalyzes light-induced electron transport from 1,5-DPC to SiMo (5). These properties make the complex of considerable interest for further biochemical and photophysical studies; however, the complex is isolated at low concentration and is rather unstable (5, 21).

To address these problems, we have attempted to both concentrate and, more importantly, stabilize the complex by several methods. We found that precipitating the reaction center with PEG and resuspending it in buffer with or without small amounts of the nonionic detergent, lauryl maltoside, concentrates the reaction center material and greatly improves its stability, both in the dark and in the light. A previous paper (21) describes an earlier version of the PEG technique, and a preliminary report of the current work has appeared (16).

MATERIALS AND METHODS

Reaction center was isolated according to Nanba and Satoh (16) from PSII-enriched appressed spinach (*Spinacia oleracea* L.) membrane fragments (8) prepared by the method of Kuwabara and Murata (12). The membranes were stored at −80°C until use. Thirty mL of PSII membranes (1 mg Chl/mL) were solubilized in 4% Triton X-100, 50 mM Tris-HCl (pH 7.2) for 1 h at 4°C and centrifuged at 33,000*g* for 1 h (pelleting at ultrahigh *g* values reported in Ref. 17 is not necessary). The supernatant was loaded onto a 1.6 × 15 cm anion-exchange column containing Fractogel TSK-DEAE 650S now sold by Supelco, Bellefonte, PA, under the trade name TSK-GEL DEAE-Toyopearl 650S. The column was washed with approximately 4 L of 50 mM Tris-HCl (pH 7.2), 0.05% Triton (buffer A) containing 30 mM NaCl overnight until the absorbance (1-cm pathlength) at 670 nm of the eluant was below 0.01. The reaction center material was then eluted with a 300 mL 30 to 200 mM NaCl gradient in buffer A. The green reaction center fraction, hereafter termed control reaction center, was concentrated by one of the following three techniques.

Concentration Ion-Exchange Column

Control reaction center was diluted with an equal volume of buffer A and loaded onto a second Fractogel column (1.1 × 4 cm). The column was washed with buffer A containing 30 mM NaCl and the reaction center eluted with 150 mM NaCl.

[1] Sponsored by the Division of Energy Biosciences, Office of Basic Energy Sciences, United States Department of Energy (contract No. 18-006-88) and by a grant from the Solar Energy Research Institute Director's Development Fund to M. S. H. M. is an Associated Western Universities Postgraduate Research Participant at SERI.

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[4] A division of the Midwest Research Institute operated for the U.S. Department of Energy under contract No. DE-AC02-83CH-10093.

[5] Abbreviations: Pheo, pheophytin; DPC, diphenylcarbazide; SiMo, silicomolybdate; LDS, lithium dodecyl sulfate; IE, ion exchange; UC, ultracentrifuge.

Ultracentrifugation of Highly Diluted Material

Control reaction center was diluted 1:7 with 50 mM Tris-HCl (pH 7.2) (buffer B) and centrifuged at 309,000*g* for 3 h to pellet aggregated reaction center (this procedure was suggested to us by Dr. M. Ikeuchi, The Institute of Physical and Chemical Research, Wako-shi, Japan). The pellet was resuspended in the same buffer with or without added detergent.

PEG Precipitation

To control reaction center, 0.325 g of PEG (Sigma; mol wt = 3,350) per mL was added slowly and dispersed carefully with a small paintbrush. After 90 min incubation, the suspension was centrifuged at 31,300*g* for 15 min (see also Ref. 21). The pellet was resuspended in buffer B with detergent as noted in the tables, and centrifuged again at 1,100*g* for 90 s to pellet mostly colorless material containing PEG aggregates. The exact conditions of the last centrifugation may have to be adjusted if green material appears in the pellet. All preparative procedures were done at 4°C in the dark, LDS-PAGE was performed as in Seibert *et al.* (21), and absorption spectra obtained with an HP 8450A diode array spectrophotometer.

Photochemical competence of the reaction center was assayed by photoinduced electron transport from DPC to SiMo (1) at 4°C, monitored by observing absorbance increases at 600 nm, with a Cary 17D spectrophotometer. Reaction center was diluted to 0.7 μg Chl/mL (final concentration) in 60 mM Tris-HCl (pH 8.5), 0.025% Triton. After incubation for 2 min, DPC (Sigma) was added to 43 μg/mL and SiMo (Pfaltz and Bauer, Waterbury, CT) to 200 μg/mL, and the sample was illuminated from the top using a slide projector. Actinic light was passed through a Schott RG 630 cutoff filter and a hot mirror filter (Melles Griot No. 03MHG007). The light intensity was about 800 W/m^2 at the sample. The photomultiplier was shielded with a narrow band 600 nm interference filter (Melles Griot No. 03FIV045). Activity was calculated using an extinction coefficient of 4.8 $mM^{-1} \cdot cm^{-1}$ (5) for SiMo. The photochemical activity of this material, representing the 100% values in the tables, ranged from 1350 to 2250 μmol SiMo reduced · mg $Chl^{-1} \cdot h^{-1}$, depending on the preparation. This is 5 to 11 times that previously reported for

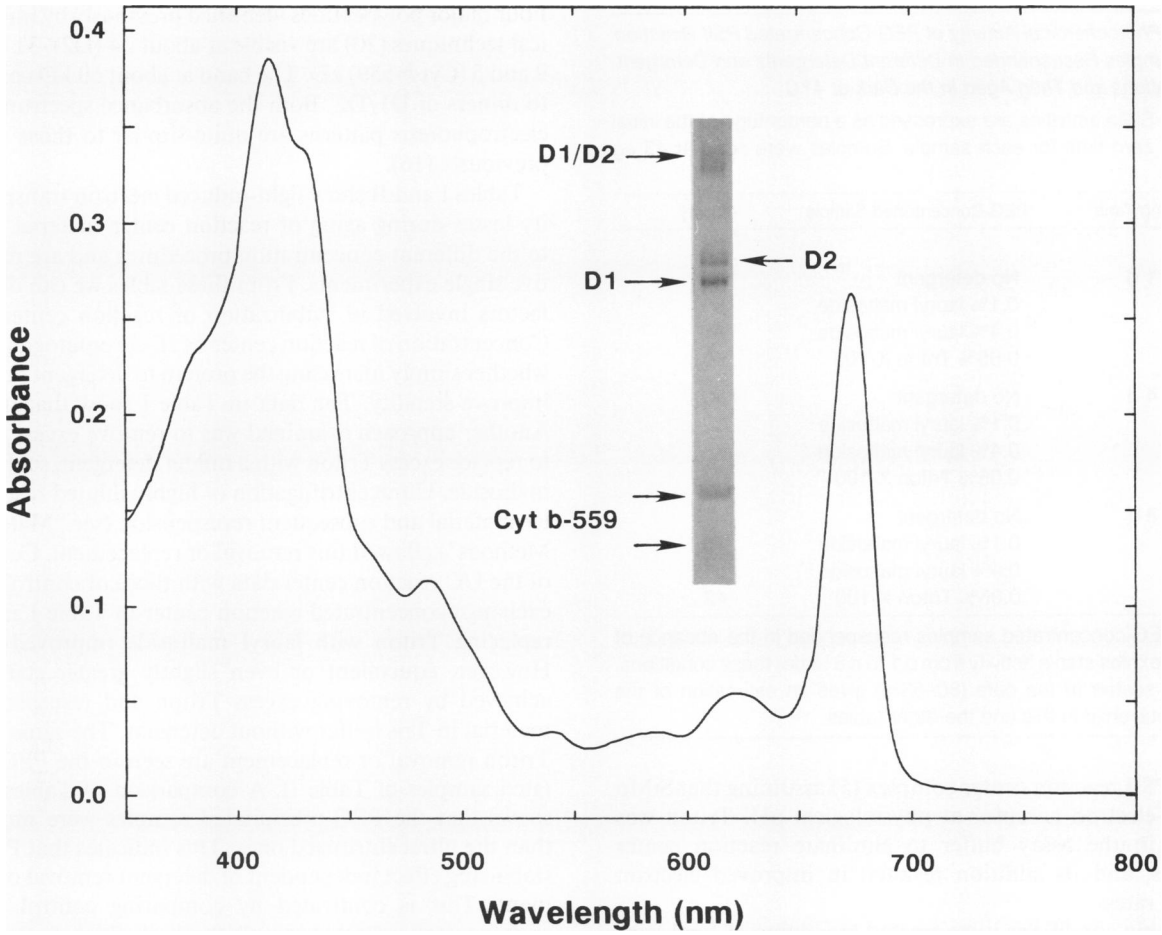

Figure 1. Absorption spectrum and LDS-PAGE profiles of PEG-concentrated PSII reaction center isolated from spinach. The spectrum was obtained after dilution in 50 mM Tris-HCl (pH 7.2). The small shoulder at about 655 nm is an instrument artifact. The LDS-PAGE (6 M urea) was run at 4°C in a 12.5% gel as in Ref. 21. Samples were denatured at room temperature for 30 min in a solution containing 50 mM Tris-HCl (pH 7.2), 2% (w/v) LDS, 2 M urea, 40 mM dithiothreitol, and 20% (w/v) sucrose. The gel was stained with Coomassie brilliant blue. D1, D2, D1/D2, and Cyt *b*-559 are discussed in the text.

Table I. *Photochemical Activity of PSII Reaction Center Complex during Aging in the Dark at 4°C*

Control reaction center material (off the first column), reaction center concentrated by a second IE column, and reaction center concentrated by UC are compared. Control and IE samples contained 0.05% Triton X-100. The UC samples were resuspended in Tris buffer with or without lauryl maltoside as indicated in parentheses. Activities are expressed as a percentage of the initial DPC to SiMo activity at zero time for each sample (see "Materials and Methods").

Aging Time	Sample	Activity
		%
6 h	Control RC[a]	79
	IE[b]	76
1 d	Control RC	56
	IE	57
	UC (no detergent)[c]	77
	UC (0.1% lauryl maltoside)[c]	79
4 d	Control RC	38
	IE	39
	UC (no detergent)	56
	UC (0.1% lauryl maltoside)	46

[a] Chl = 4.95 μg/mL during aging. [b] Chl = 26 μg/mL during aging. [c] Chl = 26.5 μg/mL during aging.

Table II. *Photochemical Activity of PEG-Concentrated PSII Reaction Center Samples Resuspended in Different Detergents and Detergent Concentrations and Then Aged in the Dark at 4°C*

DPC to SiMo activities are expressed as a percentage of the initial activity at zero time for each sample. Samples were aged at 43 μg Chl/mL.

Aging Time	PEG-Concentrated Sample	Activity
		%
1 d	No detergent	83[a]
	0.1% lauryl maltoside	88
	0.4% lauryl maltoside	81
	0.05% Triton X-100	66
4 d	No detergent	80[a]
	0.1% lauryl maltoside	82
	0.4% lauryl maltoside	75
	0.05% Triton X-100	53
8 d	No detergent	81[a]
	0.1% lauryl maltoside	76
	0.4% lauryl maltoside	66
	0.05% Triton X-100	42

[a] The PEG-concentrated samples resuspended in the absence of detergent exhibit stable activity from d 1 to d 8 under these conditions. Thus, the scatter in the data (80–83%) gives an estimation of the experimental error in this and the other tables.

isolated PSII reaction center complex (5) assuming that SiMo is a four-electron acceptor at physiological pH. Triton was required in the assay buffer to eliminate reaction center aggregates, and its addition resulted in improved electron transport rates.

Anaerobic conditions were created by adding at final concentration 20 mM glucose, 0.039 mg/mL catalase (Sigma C-100, EC 1.11.1.6), and 0.1 mg/mL glucose oxidase (Sigma G-6125, EC 1.1.3.4, type II) in that order to the samples under an argon atmosphere.

Table III. *Stability of Control PSII Reaction Center and of PEG-Concentrated PSII Reaction Center Resuspended in Tris Buffer without Detergent in the Dark and Light at 22°C*

Samples were kept in the dark or illuminated (8.5 W/m^2 from a cool-white fluorescent tube) for 45 min. Activities are expressed as a percentage of the initial DPC to SiMo activity at zero time for each sample. The Chl concentration of the samples during aging was 6.1 μg/mL.

Sample	Treatment	Activity
		%
Control RC	Aerobic in the dark	44
	Aerobic in the light	16
	Anaerobic in the light	29
PEG-concentrated	Aerobic in the dark	92
	Aerobic in the light	46
	Anaerobic in the light	93

RESULTS AND DISCUSSION

The absorption spectrum of PEG-precipitated PSII reaction center (Fig. 1) shows major peaks and shoulders at 674, 622, 540, 484, 434, and 414 nm in the visible region. Figure 1 also shows a 6 M urea LDS-PAGE run of the same preparation. Four major polypeptides identified previously by immunological techniques (20) are visible at about 34 (D2), 31 (D1), and 9 and 5 (Cyt *b*-559) kD. The band at about 60 kD corresponds to dimers of D1/D2. Both the absorbance spectrum and the electrophoresis patterns are quite similar to those published previously (16).

Tables I and II show light-induced electron transport activity losses during aging of reaction center material subjected to the different concentration procedures and are representative single experiments. From these tables we can deduce the factors involved in stabilization of reaction center activity. Concentration of reaction center by IE chromatography tested whether simply increasing the protein to detergent ratio would improve stability. The data in Table I show that it did not. Another approach examined was to remove excess Triton or to replace excess Triton with a milder detergent, such as lauryl maltoside. Ultracentrifugation of highly diluted reaction center material and subsequent resuspension (see "Materials and Methods") allowed this removal or replacement. Comparison of the UC reaction center data with those of control and ion-exchange concentrated reaction center in Table I shows that replacing Triton with lauryl maltoside improved stability. However, equivalent or even slightly greater stability was achieved by removing excess Triton and resuspending the material in Tris buffer without detergent. The same effects of Triton removal or replacement are seen in the PEG-precipitated samples of Table II. A comparison of Tables I and II shows that the PEG-precipitated samples were more stable than the ultracentrifuged ones. This indicates that PEG has a stabilizing effect independent of detergent removal or replacement. This is confirmed by comparing control and ion-exchange-concentrated sample (both of which contain 0.05% Triton) data with the data for PEG-concentrated sample resuspended in 0.05% Triton. The stabilizing effects of PEG and those of detergent removal combined to give our most stable preparations, PEG-precipitated reaction center resus-

pended in Tris buffer without detergent or with small amounts (0.1%) of lauryl maltoside (Table II).

It should be noted that comparison of the data in Tables I and II could be affected by the fact that samples were aged at different Chl concentrations. However, the control and ion-exchange-concentrating column data (Table I) indicate that reaction center concentration is not very important for stability during the aging period. We also found that storage of PEG-precipitated PSII reaction center samples, resuspended without detergent at concentrations from 8 to 73 μg Chl/mL, had no effect on stability under the conditions of Table II (data not shown).

Precipitation of PSII reaction center with PEG decreased the photochemical activity of the preparation by about 10% compared to control samples. However, this small apparent loss in activity may be an artifact of the assay caused by some PEG-induced aggregates of reaction center remaining during the assay. As mentioned earlier, the assay buffer contained 0.025% Triton which was necessary to obtain maximum electron transport rates. We assume that Triton breaks down the reaction center aggregates, favoring the interaction of the electron donor (DPC) and acceptor (SiMo) with the reaction center. Self-aggregation of proteins induced by PEG has been reported in the literature (13, 18). The other methods of concentration used in this study, ion-exchange chromatography and ultracentrifugation of diluted material, also caused little loss of photochemical activity. In any case, we emphasize that the small losses due to concentration procedure are distinct from the aging losses noted in the tables.

The stabilizing effect of replacing a harsh detergent by a mild detergent reported here has also been demonstrated with bacterial reaction centers (11) and O_2-evolving core complex from cyanobacteria (4). Also, the present use of PEG adds to the list of previous applications of this compound to precipitate proteins (10) and to stabilize enzyme activity (9, 15).

Control reaction center was unstable in the dark and even more so in the light at 22°C (Table III, see also Ref. 5). The experiment in Table III also shows that PEG precipitation of reaction center improved stability both in the dark and in the light at this temperature. However, exposure to anaerobic conditions during illumination further improved the protection of reaction center against photodestruction, virtually eliminating it in PEG-precipitated samples under these conditions. Prevention of photodestruction under anaerobic conditions strongly suggests that some photoproduct(s) of oxygen mediates the destruction. Singlet oxygen and/or superoxide radical are probable candidates. It is well known that porphyrins (2, 14, 23) are very efficient photogenerators of these destructive agents. Surprisingly, PSII reaction center seems to be more sensitive to photodamage effects than bacterial reaction center (3), despite the presence of the same amount of carotenoid in both reaction center species. Current studies in our laboratory are aimed at understanding in more detail the mechanism of the observed photodestruction of PSII reaction center. Finally, the development of effective means to stabilize the PSII reaction center reported in this paper should facilitate future photophysical, biochemical, and structural studies of the complex. For example, PEG-precipitated PSII reaction center, illuminated under anaerobic conditions as described in this paper, was stable enough to allow prolonged exposure to 500 femtosecond laser flashes necessary to measure the risetime of P^+_{680} at 4°C (24).

ACKNOWLEDGMENTS

The authors would like to thank D. Cohen for preparing PSII membrane fragments and Dr. M. Ikeuchi for helpful discussions.

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